

Uptake of 3,3',5,5'-Tetraiodothyroacetic Acid and 3,3',5'-Triiodothyronine in Cultured Rat Anterior Pituitary Cells and Their Effects on Thyrotropin Secretion*

MARIA E. EVERTS†, THEO J. VISSER, ELLIS P. C. M. MOERINGS,
ANITA M. P. TEMPELAARS, HANS VAN TOOR, ROELOF DOCTER,
MARION DE JONG, ERIC P. KRENNING, AND GEORG HENNEMANN

Departments of Internal and Nuclear Medicine, Erasmus University Medical School, 3000 DR
Rotterdam, The Netherlands

ABSTRACT

We compared the uptake, metabolism, and biological effects of tetraiodothyroacetic acid (Tetrac) and rT_3 in anterior pituitary cells with those of T_4 and T_3 . Cells were isolated from adult male Wistar rats and cultured for 3 days in medium with 10% fetal calf serum. Uptake was measured at 37°C in medium with 0.1% BSA for [125 I]Tetrac (200,000 cpm; 240 pM) and [125 I] T_4 (100,000 cpm; 175 pM) or with 0.5% BSA for [125 I] rT_3 (100,000 cpm; 250 pM) and [125 I] T_3 (50,000 cpm; 50 pM). The free fraction of Tetrac was 1% that of T_4 (in medium with 0.1 and with 0.5% BSA), and the free fraction of rT_3 was half that of T_3 . Uptake of the four tracers increased sharply up to 1 h of incubation and then leveled off. Expressed as femtomoles per pM free hormone, uptake at equilibrium was 1.16 ± 0.16 (n = 6) for Tetrac, 0.15 ± 0.01 (n = 6) for T_4 , 0.023 ± 0.003 (n = 6) for rT_3 , and 0.21 ± 0.02 (n = 6) for T_3 . Cell-associated radioactivity after incubation for 24 h with

[125 I]Tetrac was represented for 15% by [125 I]Triac; after incubation with [125 I] T_4 for 15–20% by [125 I] T_3 , after incubation with [125 I] rT_3 for 6% by [125 I]3,3'- T_2 , while [125 I] T_3 was still for 98% [125 I] T_3 . Exposure of cells for 2 h to 100 nM TRH stimulated TSH release by 90–135%. Tetrac was effective in reducing this response at a free concentration of 0.05 pM, but rT_3 was effective only at a free concentration of 16 nM. A free Tetrac concentration of 5 pM was equally effective as 50 pM free T_4 in reducing the TSH response to TRH. In human serum, Tetrac was exclusively bound to T_4 -binding prealbumin. The free Tetrac fraction was 0.001% in control subjects and rose 2- to 12-fold in patients with nonthyroidal illness. As uptake of [125 I]Tetrac in the pituitary was higher than that of T_4 and T_3 , and it was more potent than T_4 in reducing TSH release, Tetrac may be of potential significance for the regulation of TSH secretion *in vivo*. (*Endocrinology* 136: 4454–4461, 1995)

NONTHYROIDAL illness (NTI) and fasting are characterized by a low serum T_3 concentration and an elevated serum concentration of rT_3 , whereas the serum T_4 concentration is normal or eventually decreased (1–4). In the face of the low serum T_3 and T_4 concentrations, the serum TSH level is inappropriately low (1, 2, 4, 5).

Recently, it was proposed that changes in T_4 metabolism during fasting and NTI, *i.e.* a shift from deiodination to increased conjugation or alanine side-chain alteration, may result in metabolites with a TSH-suppressing effect. The first to be evaluated was T_3 sulfate (6), but this metabolite appeared to be biologically inactive at the pituitary level (7, 8), perhaps because it does not enter the tissue (8). The second to be tested was the acetic acid derivative of T_3 , triiodothyroacetic acid (Triac) (9, 10), which is rapidly taken up by the pituitary (11) and has a profound TSH-suppressing effect both *in vitro* (11, 12) and *in vivo* (13, 14). Like Triac, the acetic acid derivative of T_4 , tetraiodothyroacetic acid (Tetrac), was suggested to contribute to the suppression of TSH secretion

during fasting and NTI (10). Administration of Tetrac reduced the TSH response to TRH in euthyroid subjects (13). Furthermore, in fasting subjects, the serum Tetrac concentration was reported to increase 2-fold (15).

Although rT_3 is not ascribed any biological activity, recent studies indicate that one of its metabolites 3,3'-diiodothyronine (3,3'- T_2) stimulates the mitochondrial respiratory rate (16). A similar action has been reported for 3,5- T_2 (a product of T_3 deiodination) (16, 17), which, in addition, has been shown to suppress TSH secretion in rats (18). The pituitary contains the enzyme that deiodinates rT_3 (19), but before this can occur, rT_3 has to enter the pituitary cells.

The present study was undertaken to investigate 1) the uptake and metabolism of [125 I]-labeled rT_3 and Tetrac in cultured anterior pituitary cells compared to that of [125 I]-labeled T_3 and T_4 ; 2) the effects of unlabeled Tetrac and rT_3 on the uptake of [125 I] T_3 ; and 3) the effects of unlabeled Tetrac, rT_3 , 3,3'- T_2 , and 3,5- T_2 on TRH-induced TSH release. Finally, we evaluated the distribution of Triac and Tetrac among serum proteins as well as the free fraction of Triac and Tetrac in serum of control subjects and NTI patients.

Materials and Methods

Materials

All solutions used for cell isolation and cell culture were obtained from GIBCO Europe (Breda, The Netherlands), with the exception of human serum albumin (Central Laboratory of the Red Cross Blood

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Address all correspondence and requests for reprints to: Maria E. Everts, Ph.D., Department of Internal Medicine III, Erasmus University Medical School, Room Bd 240, Postbox 1738, 3000 DR Rotterdam, The Netherlands.

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Transfusion Service, Amsterdam, The Netherlands), dispase (grade II; Boehringer Mannheim, Mannheim, Germany), and fungizone (Bristol-Myers Squibb, Woerden, The Netherlands). Culture dishes (48 wells) were obtained from Costar (Cambridge, MA). TRH was obtained from Hoechst (Frankfurt am Main, Germany). All iodothyronines, 3,5-diiodo-thyroacetic acid (3,5-Diac), Triac, and Tetrac, were obtained from Henning Berlin (Berlin, Germany). Piperazine- N,N' -bis-[2-ethane sulfonic acid] (PIPES), HEPES, N,N -bis-[2-hydroxyethyl]2-aminoethane sulfonic acid (Bes), 6-propyl-2-thiouracil (PTU), and BSA (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). $[3',5'-^{125}\text{I}]T_4$ (1500 $\mu\text{Ci}/\mu\text{g}$), $[3'-^{125}\text{I}]T_3$ (3070 $\mu\text{Ci}/\mu\text{g}$), $[3',5'-^{125}\text{I}]rT_3$ (1200 $\mu\text{Ci}/\mu\text{g}$), and carrier-free Na^{125}I were purchased from Amersham International (Aylesbury, UK). HPLC pure $[3'-^{125}\text{I}]T_3$ (2730 $\mu\text{Ci}/\mu\text{g}$) and $[3',5'-^{125}\text{I}]T_4$ (2200 $\mu\text{Ci}/\mu\text{g}$) were prepared from Na^{125}I and 3,5-Diac or Triac, respectively, using the chloramine-T method followed by purification on Sephadex LH-20 (20). Sephadex LH-20 and G-25 were obtained from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available.

Animals

All experiments were performed using male Wistar rats, weighing 220–250 g. The animals had free access to food and water and were kept in a controlled environment (21 °C) with constant day length (12 h).

Cell culture

Animals (12–18 for each experiment) were killed between 0900–0930 h by decapitation. The pituitary glands were removed within 5 min, the neurointermediate lobe was discarded, and the anterior lobes were collected in calcium- and magnesium-free Hanks' Balanced Salt Solution supplemented with 10 g/liter human serum albumin, penicillin (10^5 U/liter), fungizone (0.5 mg/liter), and sodium bicarbonate (0.4 g/liter). Anterior pituitary cells were dissociated with dispase (final concentration, 2.4×10^3 U/liter) for 2 h at 37 °C in a shaking incubator. From each pituitary, around 1.5×10^6 cells were obtained, and the viability of the cells, as determined by trypan blue exclusion, was greater than 90%.

The cells were cultured at 37 °C in a water-jacketed incubator with 5% CO_2 at a density of 500,000 cells/well in 48-well culture dishes. The cells had attached to the wells after 2 days of culture. On day 3, the cells were used for experiments. The culture medium consisted of Minimal Essential Medium with Earle's salts supplemented with nonessential amino acids, sodium pyruvate (1 mmol/liter), 10% fetal calf serum, penicillin (10^5 U/liter), fungizone (0.5 mg/liter), L-glutamine (2 mmol/liter), and sodium bicarbonate (2.2 g/liter), pH 7.4 (21–23).

Cellular uptake of ^{125}I -labeled rT_3 , T_3 , Tetrac, and T_4

After removal of the culture medium, cells were preincubated with 0.5 ml incubation medium for 30 min at 37 °C. The incubation medium was identical to the culture medium, except that fetal calf serum was replaced by 0.5%, 0.1%, or 0.01% BSA. After preincubation the medium was removed, and incubation (15 min–4 h) was started with 0.25 ml medium containing $[^{125}\text{I}]rT_3$ (100,000 cpm; 250 pM), $[^{125}\text{I}]T_3$ (50,000 cpm; 50 pM), $[^{125}\text{I}]T_4$ (100,000 cpm; 175 pM), or $[^{125}\text{I}]T_4$ (200,000 cpm; 240 pM). Incubations lasting for more than 1 h were performed at 37 °C in humidified air with 5% CO_2 . Incubations of shorter duration took place in a 37 °C incubation chamber on a rotating device without CO_2 . Therefore, the NaHCO_3 in the culture medium was replaced by an equimolar amount of HEPES (8.9 mM), PIPES (10.6 mM), and Bes (11.2 mM). After incubation, the medium was removed, and cells were washed with 1-ml volumes of ice-cold saline to remove tracer not bound to the cells. Cells were dissolved in 1 ml 0.1 N NaOH and counted for ^{125}I activity in a 16-channel γ -counter (NE 1600, Nuclear Enterprises, Edinburgh, Scotland). The amount of ^{125}I -labeled compound taken up was expressed as a percentage of the radioactivity added (percent dose). The same procedure was applied to the incubations without cells. All results are corrected for the amount of radioactivity retained in the wells without cells.

For HPLC analysis of metabolites, incubations were performed for 24 h with 2×10^6 cpm T_3 (2 nM), T_4 (3.5 nM), rT_3 (5 nM), or Tetrac (2.4 nM). Cells were further processed as described above.

LH-20 chromatography

Aliquots of the incubation medium and cell extracts were chromatographed on Sephadex LH-20 (24). Iodide was eluted from the column with 4×1 ml 0.1 N HCl. Subsequently, conjugates were eluted with 8×1 ml H_2O , and finally, iodothyronines were removed from the column with 3×1 ml 1% NH_4OH in ethanol.

HPLC

The solvent (1% NH_4OH in ethanol) of samples prepurified on Sephadex LH-20 was evaporated under a stream of N_2 at 50 °C. The residue was redissolved in the HPLC mobile phase and injected onto Chromspher C_{18} columns (10×0.3 cm; Chrompack International, Middelburg, The Netherlands) fitted in a Waters HPLC system (Waters Associates, Milford, MA). Isocratic elution was performed with a 53:47 mixture of methanol in 0.02 M ammonium acetate (pH 4) at a flow rate of 0.9 ml/min for studies of $[^{125}\text{I}]T_4$ metabolism and with a 60:40 mixture of the same solutions at the same flow rate for studies of $[^{125}\text{I}]T_4$ metabolism. The latter was performed to speed up elution of the acetic acid derivatives. Fractions of 0.3 min were collected and counted for radioactivity. The retention times of the four unlabeled reference compounds were determined with the two elution mixtures by monitoring the absorbance of the eluate at 254 nm (20). The procedure was applied to cell samples as well as to the blank samples (without cells) to allow correction for degradation of $[^{125}\text{I}]T_4$ or $[^{125}\text{I}]T_4$ during evaporation and HPLC analysis.

TSH release

After removal of culture medium, cells were washed once with incubation medium. The incubation medium was identical to the culture medium, except that the fetal calf serum was replaced by 0.5% BSA. Pituitary cells were preincubated (0.5 ml) for 2 h at 37 °C in a water-jacketed incubator with 5% CO_2 in the absence or presence of variable concentrations (0.01 nM to 1 μM) of T_3 , T_4 , rT_3 , Triac, Tetrac, 3,5- T_2 , or 3,3'- T_2 . The preincubation medium was discarded, and fresh incubation medium (0.5 ml) was added containing TRH (100 nM) without or with the same additions as described above. Incubation was continued for 2 h at 37 °C. This medium was removed, centrifuged ($2000 \times g$), and stored at –20 °C. TSH in incubation medium was measured by RIA (22).

Free fractions of iodothyronine derivatives

The free fractions of iodothyronines, Tetrac and Triac, were determined by equilibrium dialysis (25). The free Triac fraction was corrected for the fact that around 70% of $[^{125}\text{I}]T_3$ precipitated with MgCl_2 . The free fractions of all iodothyronines used in this study were determined in incubation medium with 0.5% BSA; those of Tetrac and T_4 were also determined in incubation medium with 0.1% BSA. The free fractions of Triac and Tetrac were also determined in the serum of five control subjects and six NTI patients.

Agar gel electrophoresis

The distribution of Triac and Tetrac among serum binding proteins was determined by agar gel electrophoresis (26). The sera (50 μl) were mixed with bromophenol blue (1 μl) and ^{125}I -labeled Triac or Tetrac (10^6 cpm). Gels were run for 60 min at 70 mA/slide (~ 150 V) at 15 °C. Successive slices of 2 mm gel were counted, and the binding proteins T_4 -binding globulin (TBG), albumin, and T_4 -binding prealbumin (TBPA) were identified using the bromophenol blue staining of albumin as a reference; the region between the origin and albumin contained TBG, whereas the region anodal to albumin contained TBPA.

Statistics

The statistical significance of any of the tested compounds on $[^{125}\text{I}]T_3$ uptake or on TSH secretion was evaluated by Student's t test or one-way analysis of variance and Duncan's test for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

Results

Free fractions of iodothyronine derivatives

The free fractions of 3,3'- T_2 , T_3 , rT_3 , T_4 , Triac, and Tetrac in medium with 0.5% BSA are shown in Fig. 1. The free fraction decreases as the number of iodine atoms in the compound increases, whereas a change in the alanine side-chain to acetic acid, as in Triac and Tetrac, decreases the free fraction by a factor 8 (Triac vs. T_3) or 100 (Tetrac vs. T_4). The free fractions of Tetrac and T_4 in medium with 0.1% BSA amounted to $0.029 \pm 0.001\%$ ($n = 3$) and $3.33 \pm 0.08\%$ ($n = 4$), respectively, i.e. again a 100-fold difference. The free T_3 fraction ($3.61 \pm 0.05\%$; $n = 12$) did not change after the addition of 1 nM – $1 \mu\text{M}$ T_3 , Triac, Tetrac, or rT_3 , but it increased to $4.53 \pm 0.13\%$ ($n = 4$), 4.51% ($n = 2$), $4.17 \pm 0.02\%$ ($n = 4$), and $4.43 \pm 0.09\%$ ($n = 4$) after the addition of $10 \mu\text{M}$ T_3 , Triac, Tetrac and rT_3 , respectively.

Time course of uptake of [^{125}I] rT_3 and [^{125}I]Tetrac

Figure 2 shows the time course of [^{125}I] rT_3 uptake compared with that of [^{125}I] T_3 expressed as a percentage of the dose (Fig. 2A) or as femtomoles per pM free hormone (Fig. 2B). The uptake curve for [^{125}I] T_3 was similar to that previously described (11, 22), showed a steep phase up to 1 h of incubation, and reached equilibrium between 1–4 h of incubation (Fig. 2A). At any time point of the experiment, the uptake of [^{125}I] rT_3 was only 3–4% of the uptake of [^{125}I] T_3 (Fig. 2A, lower curve). When expressed as femtomoles per pM free hormone, there was still a large difference between the uptake of the two hormones, but as the free fraction of rT_3 was half that of T_3 , the values for rT_3 uptake were about 10% of those for T_3 uptake (Fig. 2B).

Similar comparisons were made for the uptake of [^{125}I]Tetrac and [^{125}I] T_4 in incubation medium with 0.1% BSA (Fig. 3). Uptake of [^{125}I] T_4 showed a steep phase up to 1 h of incubation and reached a plateau at 4 h of incubation (Fig. 3A, upper curve). Uptake of [^{125}I]Tetrac, expressed as percent dose, was less than 0.15% at any time point of the incubation (Fig. 3A, lower curve), and [^{125}I] T_4 uptake was about 10-fold higher at equilibrium (Fig. 3A, upper curve). However, when the data were expressed as femtomoles per pM free hormone, the picture was precisely reversed; the maximum uptake of [^{125}I]Tetrac was 1.16 ± 0.16 ($n = 6$) fmol/pM free Tetrac, and

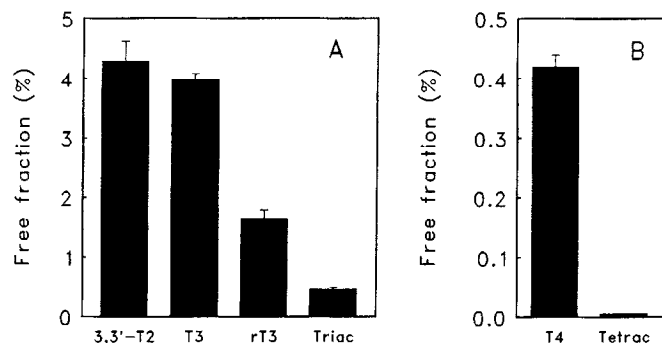


FIG. 1. Free fraction of iodothyronines and analogs in incubation medium with 0.5% BSA. Bars show the mean \pm SE of three to eight determinations of the free fraction by equilibrium dialysis. A and B differ by a factor of 10 in the scale on the y-axis.

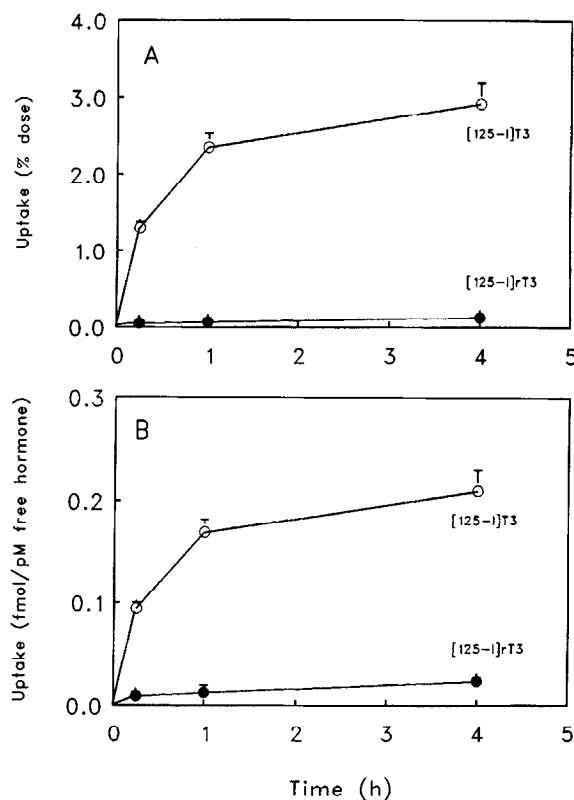


FIG. 2. Time course of uptake of [^{125}I] T_3 (\circ) and [^{125}I] rT_3 (\bullet) in cultured anterior pituitary cells expressed as the percent dose (A) or as femtomoles per pM free hormone (B). Cells were cultured for 3 days at a density of 500,000 cells/well. After preincubation for 30 min in incubation medium with 0.5% BSA, cells were incubated for 15 min, 1 h, or 4 h in the presence of [^{125}I] T_3 (50,000 cpm) or [^{125}I] rT_3 (100,000 cpm). Data represent the mean \pm SE of six observations from two independent experiments.

that of [^{125}I] T_4 was 0.15 ± 0.01 ($n = 6$) fmol/pM free T_4 ($P < 0.001$; Fig. 3B).

Metabolism of iodothyronines and Tetrac

Table 1 shows the presence of iodide and conjugates in the incubation medium (first two columns) and HPLC analysis of the cell-associated radioactivity (last four columns) after incubation of pituitary cells with [^{125}I] T_3 , [^{125}I] T_4 , and [^{125}I] rT_3 for 24 h. After incubation with [^{125}I] T_3 , no iodide was detectable with only a small amount of conjugates. Furthermore, almost all cell-associated radioactivity represented [^{125}I] T_3 , indicating that T_3 is not metabolized in pituitary cells. Within the same experiment, however, [^{125}I] T_4 was metabolized, as shown by the production of iodide in the medium and the presence of [^{125}I] T_3 in the cells, representing 20% of the cell-associated radioactivity. The conversion of [^{125}I] T_4 to [^{125}I] T_3 was not inhibited by simultaneous incubation with $100 \mu\text{M}$ PTU, but was almost completely blocked by the presence of $1 \mu\text{M}$ unlabeled T_4 . Finally, incubation with [^{125}I] rT_3 resulted in a significant production of iodide and conjugates, whereas about 6% of the cell-associated radioactivity represented [^{125}I] T_2 .

The 24-h uptake and metabolism of [^{125}I]Tetrac were compared with those of [^{125}I] T_4 (Table 2). To ensure that uptake

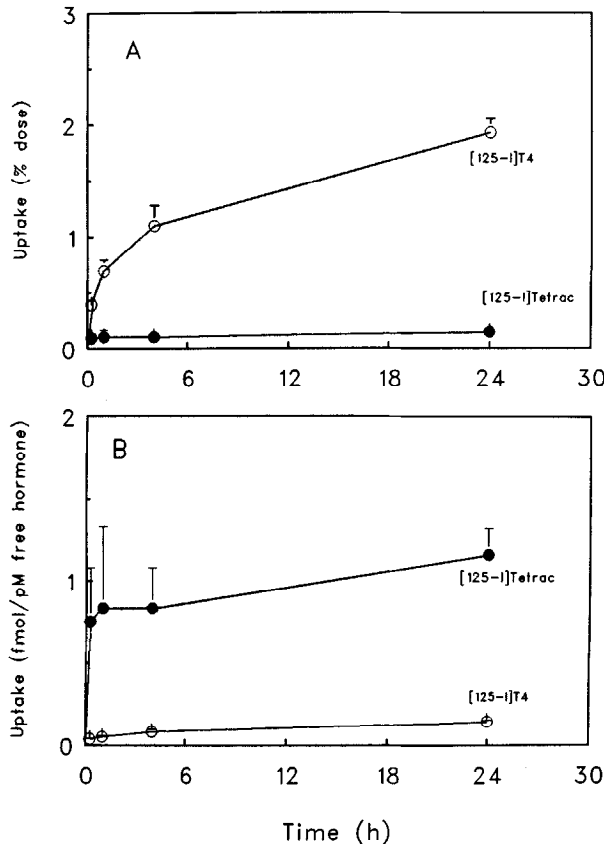


FIG. 3 Time course of uptake of $[^{125}\text{I}]\text{T}_4$ (○) and $[^{125}\text{I}]\text{Tetrac}$ (●) in cultured anterior pituitary cells expressed as the percent dose (A) or as femtomoles per pM free hormone (B). Cells were cultured and the experiment was performed as described in Fig. 2, except that the incubation medium contained 0.1% BSA and $[^{125}\text{I}]\text{T}_4$ ($0.1\text{--}2 \times 10^6$ cpm) or $[^{125}\text{I}]\text{Tetrac}$ ($0.2\text{--}2 \times 10^6$ cpm). Data represent the mean \pm SE of six observations from two independent experiments.

of $[^{125}\text{I}]\text{Tetrac}$ was large enough to allow Sephadex LH-20 chromatography and HPLC, the experiment was carried out with 0.01% and 0.1% BSA in the incubation medium. As shown in Table 2, about 15% of the cell-associated radioactivity of $[^{125}\text{I}]\text{T}_4$ and $[^{125}\text{I}]\text{Tetrac}$ represented $[^{125}\text{I}]\text{T}_3$ and $[^{125}\text{I}]\text{Triac}$, respectively, after incubation for 24 h under the two experimental conditions. Net release of $[^{125}\text{I}]\text{iodide}$ into the medium was: in 0.01% BSA, 3.9% ($n = 2$) from $[^{125}\text{I}]\text{T}_4$ and $1.8 \pm 0.2\%$ ($n = 3$) from $[^{125}\text{I}]\text{Tetrac}$; and in 0.1% BSA, $2.8 \pm 0.3\%$ ($n = 3$) from $[^{125}\text{I}]\text{T}_4$ and $0.6 \pm 0.1\%$ ($n = 3$) from $[^{125}\text{I}]\text{Tetrac}$.

Effects of rT_3 , Tetrac, and Triac on $[^{125}\text{I}]\text{T}_3$ uptake

Due to the very low tracer uptake (percent dose) of $[^{125}\text{I}]\text{Tetrac}$ and $[^{125}\text{I}]\text{rT}_3$, it was impossible to perform detailed studies on the effects of thyroid hormone analogs or metabolic inhibitors on the plasma membrane uptake of these tracers. Therefore, we studied the effects of unlabeled Tetrac and Triac on the time course of $[^{125}\text{I}]\text{T}_3$ uptake (Fig. 4) as well as the effects of unlabeled rT_3 and Tetrac on the 15-min uptake of $[^{125}\text{I}]\text{T}_3$ (Fig. 5).

The uptake of $[^{125}\text{I}]\text{T}_3$ was at any time point significantly reduced by the presence of 10 μM Tetrac or Triac, and the

effects of these analogs were similar. The effects of Tetrac and Triac were approximately 35%, 45%, and 70% after 15 min, 1 h, and 4 h of incubation, respectively (all $P < 0.001$; Fig. 4).

Measurements of $[^{125}\text{I}]\text{T}_3$ uptake in the linear part of the curve presented in Fig. 4, *i.e.* after 15 min of incubation, can be used to study uptake at the level of the pituitary plasma membrane (22, 23). The effects of Tetrac and rT_3 at concentrations from 1 nM to 1 μM on the 15-min uptake of $[^{125}\text{I}]\text{T}_3$ were compared with those of unlabeled T_3 . The effect of unlabeled T_3 increased progressively with the concentration, reaching a maximum of 68% suppression ($P < 0.05$) at 10 μM . In contrast, Tetrac and rT_3 did not affect $[^{125}\text{I}]\text{T}_3$ uptake up to a concentration of 1 μM , but reduced $[^{125}\text{I}]\text{T}_3$ uptake by about 45% ($P < 0.05$) at a concentration of 10 μM (Fig. 5).

Effects on TRH-induced TSH release

Table 3 shows the effects of Tetrac and rT_3 compared to those of Triac on TSH release during short term exposure to TRH. TSH release increased by approximately 90% after exposure to 100 nM TRH for 2 h ($P < 0.05$). This effect was significantly reduced by preincubation (2 h) and incubation with Triac at a concentration as low as 0.01 nM (71% suppression; $P < 0.05$) and was fully blocked at concentrations of 1–10 nM Triac and higher ($P < 0.05$). Preincubation and incubation with Tetrac resulted in a dose-dependent inhibition that was significant at 1 nM, and a maximum (82% inhibition; $P < 0.05$) was seen with a concentration of 1 μM . Preincubation and incubation with rT_3 showed a smaller inhibitory effect than that seen with Tetrac. Only at a rT_3 concentration of 1 μM was the TSH response to TRH significantly reduced (57%; $P < 0.05$). A direct comparison between Tetrac and T_4 in a single experiment showed that 100 nM Tetrac reduced TRH-induced TSH release to the same extent as 10 nM T_4 (36% and 27% inhibition, respectively; Table 4).

Similarly, the effects of preincubation and incubation with 3,3'- T_2 and 3,5- T_2 were compared with those of T_3 (Table 5). The stimulation during short term exposure to 100 nM TRH was 133% ($P < 0.05$) in these experiments. Preincubation and incubation with T_3 resulted in a dose-dependent inhibition of the TSH response to TRH, which was significant at concentrations of 1 nM and higher. 3,5- T_2 reduced the TSH response significantly at a dose of 1 nM, and the effect of 100 nM 3,5- T_2 was as great as that of 1 nM T_3 (55% suppression; $P < 0.05$). 3,3'- T_2 showed no inhibitory effect in doses less than 100 nM, but this dose had the same effect as 100 nM 3,5- T_2 or 1 nM T_3 .

Serum binding and free fraction of Triac and Tetrac

The binding of Triac and Tetrac to serum proteins was determined in four control subjects and four NTI patients (Table 6). In control subjects, Triac was equally bound to albumin and TBPA, but in NTI patients, more Triac was bound to albumin than to TBPA. Binding of Triac to TBG was negligible in both groups. In contrast, Tetrac was almost exclusively bound to TBPA in control subjects. Binding of Tetrac to albumin and TBG was about 5%. Also, the binding of Tetrac to TBPA was reduced in NTI patients, whereas

TABLE 1. Metabolism of [¹²⁵I]T₃, [¹²⁵I]rT₃, and [¹²⁵I]T₄ in cultured anterior pituitary cells

	Medium (%)		Cellular content (%)			
	Iodide	Conjugates	[¹²⁵ I]T ₃	[¹²⁵ I]T ₄	[¹²⁵ I]rT ₃	[¹²⁵ I]T ₂
[¹²⁵ I]T ₃ (3)	0.0 ± 0.0	0.28 ± 0.01	98.0 ± 0.1			
[¹²⁵ I]T ₄ (6)	1.10 ± 0.04	0.16 ± 0.01	21.0 ± 0.6	71.2 ± 0.3		
[¹²⁵ I]T ₄ + 100 μM PTU (3)	1.89 ± 0.03 ^a	0.16 ± 0.0	21.2 ± 0.5	70.9 ± 0.9		
[¹²⁵ I]T ₄ + 1 μM T ₄ (3)	0.44 ± 0.09 ^a	0.32 ± 0.02 ^a	1.9 ± 0.3 ^a	88.1 ± 1.8 ^a		
[¹²⁵ I]rT ₃ (3)	1.86 ± 0.15	2.12 ± 0.02			82.4 ± 1.0	6.3 ± 0.6

Data represent the mean ± SE of three to six observations in a single experiment as indicated in parentheses. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The cells were preincubated for 30 min in incubation medium containing 0.5% BSA. Then they were incubated for 24 h in the same medium containing 2×10^6 cpm of any of the tracers without or with 100 μM PTU or 1 μM T₄. After incubation, aliquots of the medium and the cells were analyzed by Sephadex LH-20 chromatography. The iodothyronine fraction from the cells was further analyzed by HPLC.

^a $P < 0.001$ vs. [¹²⁵I]T₄ alone.

TABLE 2. Metabolism of [¹²⁵I]T₄ and [¹²⁵I]Tetrac in cultured anterior pituitary cells

Substrate	Uptake (% dose)	[¹²⁵ I] activity in cells (%)	
		T ₄ or Tetrac	T ₃ or Triac
0.01 % BSA			
[¹²⁵ I]T ₄	5.69 ± 0.09	85.2 ± 0.8	12.0 ± 0.7
[¹²⁵ I]Tetrac	0.35 ± 0.08	74.5 ± 3.6	15.2 ± 1.7
0.1% BSA			
[¹²⁵ I]T ₄	2.03 ± 0.07	73.1 ± 2.5	17.8 ± 1.2
[¹²⁵ I]Tetrac	0.16 ± 0.01	79.4 ± 4.2	12.6 ± 0.4

Data represent the mean ± SE of three observations in a single experiment. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The experiment was performed as described in Table 2 with 2×10^6 cpm [¹²⁵I]T₄ or [¹²⁵I]Tetrac with BSA concentrations in the incubation medium as indicated. The product of [¹²⁵I]T₄ metabolism was identified by HPLC as T₃, and that of [¹²⁵I]Tetrac metabolism was identified as Triac.

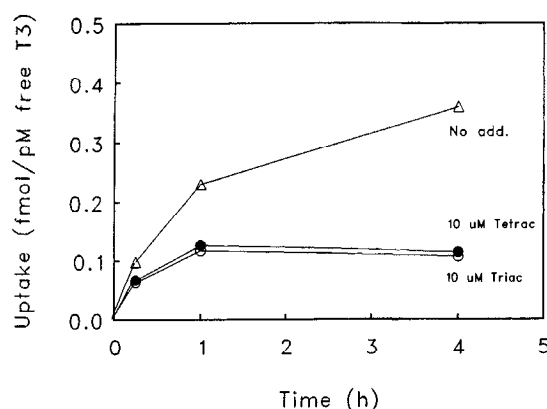


FIG. 4. Effects of unlabeled Triac or Tetrac on the uptake of [¹²⁵I]T₃ in cultured anterior pituitary cells. Cells were cultured and the experiment was performed as described in Fig. 2, except that the incubation contained [¹²⁵I]T₃ (50,000 cpm) (Δ) without or with 10 μM Triac (○) or Tetrac (●). Data represent the mean ± SE of three to six observations from two independent experiments.

more Tetrac was bound to albumin than in the control subjects (Table 6).

The changes in serum binding during NTI (Table 6) resulted in remarkable changes in the free fractions of Triac and Tetrac in NTI patients (Fig. 6). In control subjects (no. 1–5), the free Triac fraction was approximately 0.04%, whereas that of Tetrac was 0.0011%. Similarly low values for the free fractions of Triac and Tetrac in normal serum have previously been described (27). During NTI (patients 6–11), the

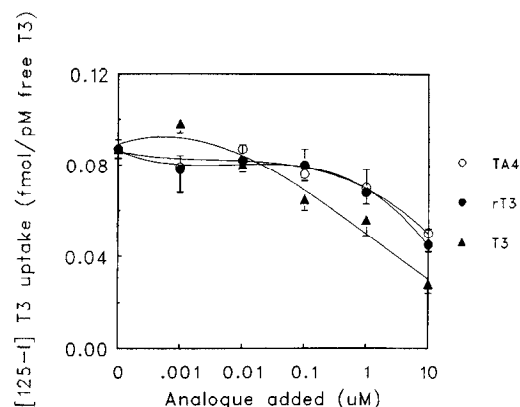


FIG. 5. Effects of unlabeled T₃ (▲), rT₃ (●), or Tetrac (○; TA₄) on the 15-min uptake of [¹²⁵I]T₃ in cultured anterior pituitary cells. Cells were cultured and uptake of [¹²⁵I]T₃ was measured for 15 min as described in Fig. 2. Incubation was performed with [¹²⁵I]T₃ (50,000 cpm) without or with unlabeled T₃, rT₃, or Tetrac present in concentrations of 1 nM to 10 μM. Each point represents the mean ± SE of six observations from two independent experiments.

free Triac fraction increased 2- to 8-fold, and that of Tetrac increased 2- to 12-fold.

Discussion

The present study shows that both [¹²⁵I]Tetrac and [¹²⁵I]rT₃ are taken up and metabolized in cultured anterior pituitary cells. In addition, Tetrac and its major metabolite Triac significantly reduced the TSH response to TRH at total hormone concentrations of 1 and 0.01 nM, respectively, whereas rT₃ and its metabolite 3,3'-T₂ had to be added at total concentrations of 1 μM and 100 nM, respectively, to obtain a significant reduction of the TRH-induced TSH release.

Expressed as percent dose, the uptake of [¹²⁵I]Tetrac and [¹²⁵I]rT₃ was very low. That both tracers were taken up by pituitary cells was indicated by the 24-h incubation experiments. Around 15% of the cell-associated radioactivity was [¹²⁵I]Triac after incubation with [¹²⁵I]Tetrac, 6% of cell-associated radioactivity was [¹²⁵I]T₂ after incubation with [¹²⁵I]rT₃, and 15–20% of cell-associated radioactivity was [¹²⁵I]T₃ after incubation with [¹²⁵I]T₄. The deiodination of [¹²⁵I]T₄ was almost completely blocked by simultaneous incubation with 1 μM unlabeled T₄, but not by 100 μM PTU. [¹²⁵I]T₃ was not metabolized in pituitary cells. Together,

TABLE 3. Effects of Tetrac, rT_3 , and Triac on TSH release from anterior pituitary cells during short term exposure to TRH

Exp conditions	TSH release (ng)	Stimulation (%)
Controls (23)	3.15 ± 0.22	
+ 100 nM TRH (25)	5.95 ± 0.14	+89 ^a
TRH + 1 nM Tetrac (3)	4.87 ± 0.26	+55 ^b
TRH + 10 nM Tetrac (6)	4.82 ± 0.32	+53 ^b
TRH + 100 nM Tetrac (3)	4.09 ± 0.39	+30 ^b
TRH + 1 μM Tetrac (9)	3.73 ± 0.19	+18 ^b
TRH + 1 nM rT_3 (3)	5.22 ± 0.64	+66
TRH + 10 nM rT_3 (6)	5.29 ± 0.18	+68
TRH + 100 nM rT_3 (3)	4.93 ± 0.43	+57
TRH + 1 μM rT_3 (6)	4.50 ± 0.11	+43 ^b
TRH + 0.01 nM Triac (3)	4.06 ± 0.35	+29 ^b
TRH + 0.1 nM Triac (3)	3.83 ± 0.25	+22 ^b
TRH + 1 nM Triac (3)	3.40 ± 0.26	+8 ^b
TRH + 10 nM Triac (9)	3.10 ± 0.19	-2 ^b
TRH + 100 nM Triac (6)	3.43 ± 0.26	+9 ^b
TRH + 1 μM Triac (6)	2.90 ± 0.17	-8 ^b

Data indicate the mean of four experiments, with the number of observations in parentheses. Each experiment included at least two of the tested compounds at comparable concentrations. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The cells were preincubated for 2 h in the absence or presence of Tetrac, rT_3 , or Triac at concentrations of 0.1 nM to 1 μM. Then, they were incubated with TRH (100 nM) for 2 h without or with the additions indicated. TSH was measured by RIA.

^a $P < 0.05$ vs. controls.

^b $P < 0.05$ vs. TRH alone.

TABLE 4. Comparison of the effects of Tetrac and T_4 on TSH release during short term exposure to TRH

Exp conditions	TSH release (ng)	Stimulation (%)
Controls (6)	2.50 ± 0.49	
+ 100 nM TRH (6)	5.93 ± 0.30	+137 ^a
TRH + 100 nM Tetrac (3)	4.09 ± 0.39	+64 ^b
TRH + 1 μM Tetrac (3)	3.60 ± 0.41	+44 ^b
TRH + 10 nM T_4 (3)	4.33 ± 0.48	+73 ^b
TRH + 100 nM T_4 (3)	4.30 ± 0.19	+72 ^b

Data indicate the mean ± SE of three to six observations in a single experiment as indicated in parentheses. Anterior pituitary cells were cultured at a density of 500,000 cells/well. The experiment was performed as described in Table 3.

^a $P < 0.05$ vs. controls.

^b $P < 0.05$ vs. TRH alone.

these results indicated the presence of type II deiodinase activity in the cultured anterior pituitary cells (19).

Because of the low tracer uptake of [¹²⁵I] rT_3 and [¹²⁵I]Tetrac, it was impossible to test the effects of metabolic inhibitors and thyroid hormone analogs on the transport process. Therefore, we tested the effects of rT_3 and Tetrac on the uptake of [¹²⁵I] T_3 . Neither rT_3 nor Tetrac had an effect on the 15-min uptake of [¹²⁵I] T_3 when added at concentrations of 1–100 nM, and both showed a significant reduction of [¹²⁵I] T_3 uptake (45%) at a concentration of 1 μM. In a previous study, the presence of 10 μM rT_3 reduced the 15-min uptake of [¹²⁵I] T_4 by 32%, whereas 10 μM Tetrac had no effect (23). As T_3 and T_4 probably share the same transport mechanism in

TABLE 5. Comparison of the effects of 3,3'- T_2 , 3,5- T_2 , and T_3 on TSH release from cultured anterior pituitary cells during short term exposure to TRH

Exp conditions	TSH release (ng)	Stimulation (%)
Controls (21)	2.78 ± 0.41	
+ 100 nM TRH (21)	6.48 ± 0.45	+133 ^a
TRH + 0.1 nM 3,3'- T_2 (6)	5.45 ± 0.36	+96
TRH + 1 nM 3,3'- T_2 (9)	4.84 ± 0.43	+74
TRH + 10 nM 3,3'- T_2 (9)	5.52 ± 0.24	+98
TRH + 100 nM 3,3'- T_2 (9)	3.98 ± 0.29	+43 ^b
TRH + 0.1 nM T_3 (5)	6.27 ± 0.52	+125
TRH + 1 nM T_3 (9)	4.04 ± 0.52	+45 ^b
TRH + 10 nM T_3 (8)	3.49 ± 0.44	+26 ^b
TRH + 100 nM T_3 (9)	3.10 ± 0.26	+12 ^b
TRH + 0.1 nM 3,5- T_2 (6)	5.14 ± 0.35	+85
TRH + 1 nM 3,5- T_2 (9)	4.52 ± 0.28	+63 ^b
TRH + 10 nM 3,5- T_2 (9)	4.47 ± 0.26	+61 ^b
TRH + 100 nM 3,5- T_2 (9)	4.03 ± 0.36	+45 ^b

Data indicate the mean ± SE of three experiments, with the number of observations in parentheses. Anterior pituitary cells were cultured at a density of 500,000 cells/well. Experiments were performed as described in Table 3.

^a $P < 0.05$ vs. controls.

^b $P < 0.05$ vs. TRH alone.

the pituitary (23, 28), one might expect that uptakes of T_3 and T_4 are equally inhibited by analogs.

The results obtained in the present and our previous study (23) do not argue against the possibility that rT_3 is taken up by the same transporter involved with T_3 and T_4 uptake. The conclusion for Tetrac is more complicated. The uptake of Tetrac per picomolar concentration of free hormone (~1 fmol) was as high as that of Triac (11). Furthermore, 10 μM Tetrac reduced the uptake of [¹²⁵I] T_3 (15 min to 4 h) to the same extent as 10 μM Triac. In our study of [¹²⁵I]Triac uptake (11), it was concluded that at least part of Triac entered the pituitary by a mechanism different from the T_3 transporter. Together, the similarities between Tetrac and Triac suggest that Tetrac enters the pituitary in the same way as Triac, i.e. at least partly by another mechanism than the T_3 / T_4 transporter.

Tetrac was effective in reducing the TSH response to TRH at a total concentration of 1 nM, i.e. at a free concentration of 0.05 pM. 100 nM Tetrac showed the same inhibitory effect as 10 nM T_4 or 0.01 nM Triac. Corrected for the difference in free fraction in medium with 0.5% BSA (Tetrac, 0.005%; T_4 , 0.5%; Triac, 0.5%), 5 pM Tetrac was thus as effective as 50 pM T_4 or 0.05 pM Triac. In a direct comparison between Triac and T_3 , it was estimated that Triac was 8 times more effective than T_3 in reducing the TSH response to TRH (11). Thus, the order of potency for reducing TSH release in pituitary cells seems to be Triac > T_3 > Tetrac > T_4 .

The question remains of whether the effect of Tetrac or T_4 on TSH release during 4 h of incubation is due to a direct effect or to an effect of their respective metabolites Triac and T_3 . In a previous study on the metabolism of [¹²⁵I] T_4 , it was found that iodide production into the medium after 6 h of incubation was about 40% of that found after 24 h (23). Assuming a similar value for Tetrac, and that a proportion-

TABLE 6. Distribution of Triac and Tetrac between serum proteins in control subjects and NTI patients

Subject no.	Triac (%)			Tetrac (%)		
	TBG	Albumin	TBPA	TBG	Albumin	TBPA
Control 1	2.9	43.3	53.0	1.8	5.9	88.7
Control 2	0	49.9	48.6	2.4	6.1	89.2
Control 3	0	48.6	49.6	3.6	4.9	89.2
Control 4	0	45.5	53.3	7.7	5.6	85.6
Mean \pm SE	0.7 ± 0.7	46.8 ± 1.5	51.1 ± 1.2	3.9 ± 1.3	5.6 ± 0.3	88.2 ± 0.9
NTI 7	0	59.0	39.7	3.0	6.2	89.2
NTI 8	0	54.1	44.8	4.3	9.2	85.7
NTI 9	1.0	68.6	29.7	2.6	10.5	83.2
NTI 11	2.0	64.3	33.3	3.8	10.5	81.2
Mean \pm SE	0.8 ± 0.5	61.5 ± 3.2^a	36.9 ± 3.4^a	3.4 ± 0.4	9.1 ± 1.0^b	84.8 ± 1.7

Data present individual values and the mean \pm SE of four healthy control subjects (no. 1–4) and four patients with NTI (no. 7–9, 11). The distribution of Triac and Tetrac among serum proteins was determined by agar gel electrophoresis.

^a $P < 0.005$, NTI vs. control subjects.

^b $P < 0.025$, NTI vs. control subjects.

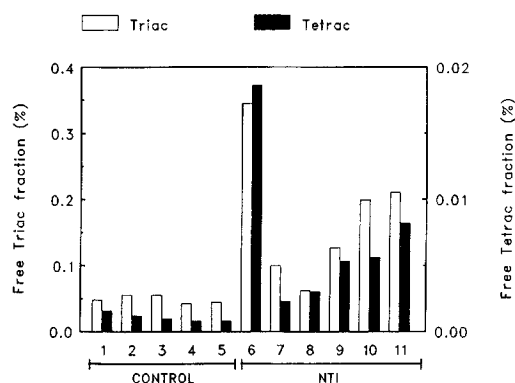


FIG. 6. Free fraction of Triac (\square ; left y-axis) and Tetrac (\blacksquare ; right y-axis) in serum obtained from five healthy control subjects (no. 1–5) and six patients with NTI (no. 6–11). The free fractions were determined in duplicate by equilibrium analysis.

ally lower amount of [125 I]Triac is formed after 6 h of incubation ($\sim 5\%$; i.e. 40% of 15% [125 I]Triac), this would mean that 10 nM Tetrac (in a volume of 0.5 ml = 5 pmol) with an uptake of 0.2% results in 0.5 fmol intracellular Triac within 6 h. Furthermore, 0.1 nM Triac (in a volume of 0.5 ml = 50 fmol), with an uptake of 1% (11) would result in 0.5 fmol intracellular Triac. As the effect of 10 nM Tetrac on TRH-induced TSH release was similar to that of 0.1 nM Triac, the effect of Tetrac on TRH-induced TSH release can be the result of its deiodination to Triac.

rT_3 was only effective at a total concentration of 1 μ M, whereas its metabolite 3,3'- T_2 induced a significant reduction in TRH-stimulated TSH release at a total concentration of 100 nM. In contrast, the T_3 metabolite 3,5- T_2 , showed a significant effect on TSH release at a concentration of 1 nM and was about 100-fold less effective than T_3 . Thus, 3,5- T_2 seems to be more effective in reducing TSH release than 3,3'- T_2 , whereas the two diiodothyronines were equally effective in stimulation of the mitochondrial respiratory rate (16). However, our results support the *in vivo* observations in rats, in which 3,5- T_2 reduced TSH secretion at doses 10- to 100-fold higher than those of T_3 (18).

The question of whether rT_3 or its metabolite 3,3'- T_2 play a role in inhibition of TSH secretion during NTI can be addressed as follows: the normal total serum levels of rT_3 and

3,3'- T_2 are 0.40 and 0.11 nM (29). The same study showed that the serum total rT_3 concentration during NTI rose to 0.66 nM, whereas that of 3,3'- T_2 declined to 0.05 nM. This should be compared with the total hormone concentrations resulting in significant effects *in vitro*, i.e. 1 μ M rT_3 and 100 nM 3,3'- T_2 , respectively, in a medium containing 0.5% BSA where the free fraction will be at least 10-fold greater than that in serum. The difference between the effective concentrations *in vitro* and those present *in vivo* is thus at least a factor of 10,000, which is not in favor of any biological significance of the two compounds *in vivo*. The small inhibitory effect of rT_3 on TRH-induced TSH release cannot solely be explained by the low uptake of rT_3 by pituitary cells; compared with T_3 , a 1000-fold higher total concentration of rT_3 was required to reduce TSH release to the same extent (1 μ M rT_3 vs. 1 nM T_3). In terms of free concentration, the difference will be a factor of 500 (free T_3 fraction, 3.8%; free rT_3 fraction, 1.9%), whereas the difference in cellular uptake was a factor of 10.

The normal total serum Tetrac concentration varies between 0.7 nM (15) and 1.3 nM (13). With a free fraction of 0.0011%, the free Tetrac concentration in normal serum will be around 0.011 pM. As the production rate increases 2-fold in fasting (15) and the mean free fraction increases 7-fold during NTI, the resulting free concentration during fasting or NTI will be about 0.15 pM. The lowest Tetrac concentration tested that reduced the response to TRH significantly was 1 nM, i.e. a free Tetrac concentration of 0.05 pM, which is of same order of magnitude as the free concentrations occurring *in vivo*.

Just as postulated for Triac, the low serum Tetrac concentration may be the result of the very effective glucuronidation in the liver (30). Although the free concentration of Tetrac in serum (0.01–0.15 pM; see above) is lower than that of Triac (0.02–0.3 pM; estimated from Refs. 9, 11, and 31), T_3 (2–4 pM) (3), or T_4 (18–25 pM) (1–3), it is not excluded that Tetrac contributes to TSH suppression during NTI, because its uptake (per picomolar concentration of free hormone) into the pituitary is much larger than that of T_3 and T_4 , whereas it is more effective than T_4 in reducing the TSH response to TRH. Also, the tentative conclusion that Tetrac (and Triac) are at least partly taken up in the pituitary by other transport mech-

animals than T_3 and T_4 would support a possible role of the compound *in vivo*.

The large rise in the estimated free concentrations of Tetrac and Triac in patients with NTI is not only due to the increase in the production rate (possibly 2- to 3-fold) (9, 15), but also to the rise in free fraction (2- to 12-fold; this study). The latter seems due to the fact that the level of TBPA, the most important binding protein for the two compounds in serum, strongly decreases during illness (10).

The conclusion of our study is that Tetrac and rT_3 , the serum concentration of which increases during fasting and NTI, are taken up and metabolized in cultured anterior pituitary cells. Only Tetrac reduces the TSH response to TRH *in vitro* at concentrations comparable to those estimated *in vivo*. Because Tetrac probably enters the pituitary through another transport system such as that described for T_3 and T_4 , our results do not exclude a potential role for Tetrac in the inappropriately low serum TSH observed during NTI.

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